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(54) **Vitamin B-12 derivatives, process for their preparation and their use in radioimmunoassays.**

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US - A - 3 981 863

CHEMICAL ABSTRACTS, vol. 82 no. 23, 9th June 1975, Columbus Ohio, USA
TORAYA, TETSU et al.: "Succinyl derivatives of vitamin B12. Preparation and biochemical properties," page 641, abstract nr. 156589c

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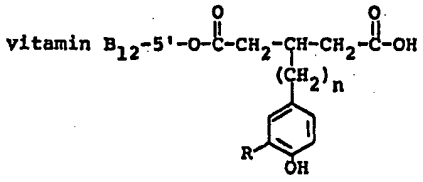
Vitamin B_{12} derivatives having the formula

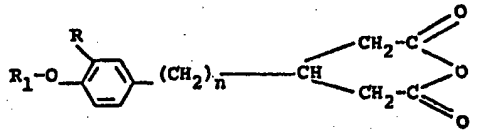


I

are readily tagged with a radioisotope and can be used (when radiolabeled) as a tracer in radioassay procedures for the determination of vitamin B_{12} levels in a body fluid. In formula I, and throughout the specification, R is hydrogen or an alkyl group of 1 to 3 carbon atoms and n is 0, 1, 2, 3, or 4.

In US—A—3,981,863 vitamin B_{12} derivatives are disclosed in which a hydroxyphenyl group is covalently bonded through one of the $—CH_2CH_2CONH_2$ or $—CH_2CONH_2$ groups of the pyrrol nuclei of the initial vitamin B_{12} molecule. These derivatives can be used in radioimmunoassays. They differ considerably from the derivatives of the present application and do not suggest the latter.

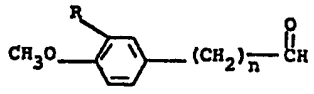
The vitamin B_{12} derivatives of formula I are prepared from vitamin B_{12} and a glutaric anhydride derivative having the formula



II

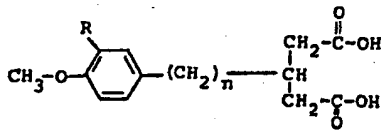
In formula II, and throughout the specification, R_1 is a phenolic hydroxyl protecting group, preferably an alkanoyl group having 2 to 6 carbon atoms, acetyl being the most preferred group.

The anhydrides of formula II are prepared by first reacting a 4-methoxyphenyl aldehyde having the formula



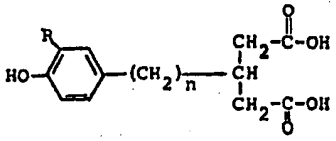
III

with at least 2 molar equivalents of cyanoacetic acid in the presence of a base (*e.g.*, a sodium hydroxide) to yield, on acid hydrolysis, a compound having the formula IV



An alternative preparation for the compound of formula IV wherein n is 0 and R is hydrogen, *i.e.*, 3-(4-methoxyphenyl)glutaric acid, is disclosed by Smith et al., *J.A.C.S., 72,* 1877 (1950). In that procedure, anisaldehyde is condensed with ethyl acetoacetate in the presence of piperidine to give ethyl anisal-bis-acetoacetate. Cleavage of this product to give the desired 3-(4-methoxyphenyl)glutaric acid can be accomplished with boiling alcoholic sodium hydroxide solution.

Demethylation of the glutaric acid derivatives of formula IV results in glutaric acid derivatives having the formula



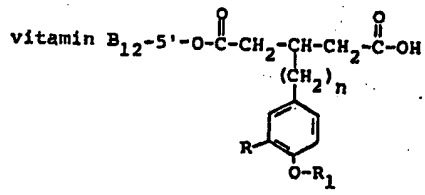
V

and can be accomplished by following one of the several procedures known in the art for the demethylation of aryl methyl ethers. One such procedure, described by Feutrill et al., *Aust. J. Chem., 25,*

1719 (1972), involves the treatment of the aryl methyl ether with thioethoxide ion (readily prepared *in situ* from ethanethiol and sodium hydride) in a polar aprotic solvent, preferably dimethylformamide.

The phenolic hydroxy group of a compound of formula V can be protected with an alkanoyl group using art-recognized procedures. One such procedure comprises reacting the glutaric acid derivative with the appropriate acid anhydride (acetic anhydride is preferred). The preferred method of preparing a glutaric anhydride derivative of formula II from the glutaric acid derivative of formula V is to combine the conversion of the acid to anhydride and the protection of the phenolic hydroxy group into a single step. When the R_1 protecting group is acetyl, this would involve heating a glutaric acid derivative of formula V in acetic anhydride.

The reaction of vitamin B_{12} and a glutaric anhydride derivative of formula II yields a compound having the formula



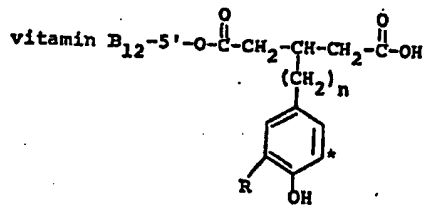
VI

the reaction can be run in the presence of an organic base. Exemplary organic bases are nitrogen containing heterocyclics, *e.g.,* pyridine, and tertiary amines, *e.g.,* triethylamine. The reaction will preferably be run at an elevated temperature.

Removal of the phenolic hydroxyl protecting group in a compound of formula VI yields the corresponding product of formula I.

The compounds of formula I can be coupled with an immunogenic carrier, such as a high molecular weight protein of which bovine serum albumin and thyroglobulin are exemplary, and if necessary an adjuvant in order to produce a substance capable of inducing antibody formation in animals. Procedures for such couplings are well known in the art; see, for example, Parker, "Radioimmunoassay of Biologically Active Compounds," Prentice-Hall, Inc., New Jersey (1976).

The compounds of formula I can be labeled ("tagged") with radioisotope, preferably iodine-125 or iodine-131, and most preferably iodine-125, using procedures well known in the art, to yield a radiolabeled compound having the formula



VII

the asterisk (*) in formula VII indicates tagging with a radioisotope. Exemplary of the methods known in the art is the method of Hunter and Greenwood; see *Nature, 194:*495 (1962). The radiolabeled compounds of formula VII form an integral part of this invention.

The vitamin B_{12} radiolabeled compounds of this invention can be used as tracers in conventional radioassays following procedures well known in the art, using hog intrinsic factor as the binding protein. Additionally, the vitamin B_{12} radiolabeled compounds of this invention can be used as tracers in radioimmunoassays following the general principles known in the art; see, for example, Parker et al., "Radioimmunoassay of Biologically Active Compounds," Prentice Hall, Inc. New Jersey (1976) or Endres, Painter, and Niswender, "A Solid-Phase Radioimmunoassay for Vitamin B_{12} in Serum. With Use of Radioiodinated Tyrosine Methyl Ester of Vitamin B_{12}", *Clin. Chem.,* 24(3):460—465 (1978). The carboxyl group on the radiolabeled compounds of this invention allows for the separation of radiolabeled tracer bound to the binding protein from unbound (free) radiolabeled tracer using ion exchange separation techniques. This feature helps make the radiolabeled compounds of this invention useful in the automated radioimmunoassay system disclosed by Brooker et al, United States patent 4,022,577.

The following examples are specific embodiments of this invention.

Example 1.

5'-O-[3-(4-Hydroxyphenyl)glutaroyl]vitamin B_{12}

A) *3-(4-Methoxyphenyl)glutaric acid*

A mixture of *p*-anisaldehyde (27.2 g), ethyl acetoacetate (52.1 g) and piperidine (4 ml) in 95% ethanol (10 ml) is stirred at room temperature for 5.0 hours while a solid forms. The solid is isolated by

filtration, washed with 25% ethanol and crystallized from 95% ethanol to afford ethyl 2,2'-(4-methoxybenzal)-*bis*-acetoacetate (31.4 g), melting point 138—141°C. The filtrate on dilution with an equal amount of water gives a solid which is crystallized from 95% ethanol to afford another crop of material (8.5 g), melting point 137—142°C.

A mixture of ethyl 2,2'-(4-methoxybenzal)-*bis*-acetoacetate (30 g), ethanol (450 ml) and 50% sodium hydroxide (450 g) is refluxed vigorously for 1.0 hour. Water (150 ml) is added and most of the ethanol is removed by distillation *in vacuo*. The concentrate is acidified with concentrated hydrochloric acid and is extracted with ethyl acetate. The ethyl acetate solution is washed with brine, dried, evaporated, and the residue is crystallized from benzene-methanol to afford 3.3 g of 3-(4-methoxyphenyl)glutaric acid, melting point 147—150°C.

B) *3-(4-Hydroxyphenyl)glutaric acid*

To a stirred suspension of 57% sodium hydride-paraffin (6.45 g), in dry dimethylformamide (70 ml) is slowly added ethanethiol (11.89 ml) in dry dimethylformamide (20 ml). After stirring the resultant slurry for 15 minutes, a solution of 3-(4-methoxyphenyl)glutaric acid (3.0 g) in dry dimethylformamide (20 ml) is added. The slurry is heated in a bath at 165°C for 5.0 hours and most of the solvent is removed by distillation *in vacuo*. The residue is diluted with water, acidified with concentrated hydrochloric acid and extracted twice with ether (the extracts are discarded). The solution is saturated with sodium chloride and extracted with ethyl acetate. The ethyl acetate solution is washed once with brine, dried, and the residue crystallized from chloroform-hexane to afford 2.3 g of 3-(4-hydroxyphenyl)glutaric acid, melting point 168—170°C.

C) *3-[(4-Acetyloxy)phenyl]glutaric anhydride*

A solution of 3-(4-hydroxyphenyl)glutaric acid (800 mg) in acetic anhydride (15 ml) is heated at 100°C for 2.5 hours and evaporated to dryness *in vacuo*. The residual solid is crystallized from chloroform-hexane to afford 600 mg of 3-(4-acetyloxyphenyl)-glutaric anhydride, melting point 140—143°C.

D) *5'-O-[3-[4-(Acetyloxy)phenyl]glutaroyl]vitamin* B_{12}

Vitamin B_{12}, 0.1012 g, is dissolved in methanol and precipitated by the addition of ethyl acetate and toluene. Removal of the solvents and drying of the residue *in vacuo* at 50°C for 30 minutes gives 100.1 mg of amorphous solid. To this solid is added, under a nitrogen atmosphere, 201 mg of 3-[(4-acetyloxy)phenyl]-glutaric anhydride (recrystallized from ethyl acetate, melting point 153—155°C), 3 ml of dimethylsulfoxide and 0.5 ml of pyridine (both the dimethylsulfoxide and pyridine are dried with type 4A molecular sieves). The resulting solution is left at room temperature in the dark for 48 hours and 116.4 mg of crude product is then precipitated by the addition of ethyl acetate.

The crude product is chromatographed on a 2.5 x 48 cm column of Whatman DE52 cellulose (acetate), eluting at 4 ml/minute with a linear gradient prepared from 2 liters of 0.1M pyridine and 2 liters of 0.2M pyridinium acetate. The effluent is monitored at 360nm and 20ml fractions are collected. Unreacted vitamin B_{12} is eluted in fractions 10 and 11, monoglutarate in fractions 45 to 54 and diglutarate (trace) fractions 76 to 100. Fractions 51 to 54 comprise a weak shoulder. Fractions 45 to 50 are combined and taken to dryness *in vacuo*. The residue is precipitated from methanol with ethyl acetate, yielding 79.1 mg of 5' - O - [3 - [4 - (Acetyloxy)phenyl]glutaroyl]vitamin B_{12}.

E) *5'-O-[3-(4-Hydroxyphenyl)glutaroyl]vitamin* B_{12}

5'-O-[3-[4-(Acetyloxy)phenyl]glutaroyl]-vitamin B_{12} (64.9 mg) is dissolved in 65 ml of water and 0.65 ml of saturated aqueous sodium bicarbonate. The resulting solution, pH 8.8., is heated at 50°C in the dark for 4 hours; the progress of the reaction is monitored by high pressure liquid chromatography. The solution is cooled to room temperature, adjusted to pH 2.0 with hydrochloric acid and applied to a 10 ml column of reverse phase adsorbent, 100 to 200 mesh macroreticular unfunctionalized divinylbenzene-cross-linked polystyrene. After washing the column with 100 ml of water, the product is eluted with methanol. The eluate is concentrated *in vacuo* and the residue, 67 mg, is chromatographed on a 2.5 x 46 cm column of DEAE-cellulose (acetate form), eluting at 4 ml/minute with a linear gradient prepared from 2 liters of 0.1*M* pyridine and 2 liters of 0.1*M* pyridinium acetate. The effluent is monitored at 550 nm and 20 ml fractions are collected. The curve obtained shows that the product, fractions 72 to 98, is not homogeneous. Fractions 72 to 98 are combined, concentrated *in vacuo*, and the residue is precipitated from methanol with ethyl acetate. The resulting solid is dissolved in water and lyophilized, yielding 60.1 mg of crude 5' - O - [3 - (4 - hydroxyphenyl) - glutaroyl]vitamin B_{12}.

Examples 2—6

Following the procedure described in Example 1D, but substituting the anhydride reagent listed in column I for 3-[(4-acetyloxy)phenyl]glutaric anhydride, yields the vitamin B_{12} derivative listed in column II.

	Column I	Column II
2.	3-[4-(acetyloxy)-3-methylphenyl] glutaric anhydride	5′-O-[3-(4-hydroxy-3-methylphenyl)glutaroyl]-vitamin B_{12}
3.	3-[[4-(acetyloxy)phenyl]-methyl]glutaric anhydride	5′-O-[3-[(4-hydroxyphenyl)-methyl]glutaroyl]vitamin B_{12}
4.	3-[2-[4-(acetyloxy)phenyl]-ethyl]glutaric anhydride	5′-O-[3-[2-(4-hydroxyphenyl)ethyl]glutaroyl] vitamin B_{12}
5.	3-[3-[4-(acetyloxy)phenyl]-propyl]glutaric anhydride	5′-O-[3-[3-(4-hydroxyphenyl)-propyl]glutaroyl] vitamin B_{12}
6.	3-[4-[4-(acetyloxy)phenyl]-butyl]glutaric anhydride	5′-O-[3-[4-(4-hydroxyphenyl)-butyl]glutaroyl]vitamin B_{12}

Detailed Procedure for Radioiodination of Vitamin B_{12} Derivative

Method 1

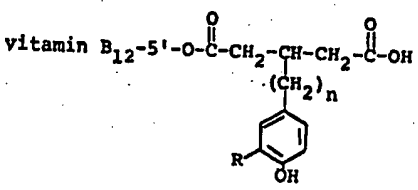
To an aqueous solution of 5′ - O - [3 - (4 - hydroxyphenyl)glutaroyl] vitamin B_{12} (20 µl; 500 µg/ml) in a glass vial is added 40 µl of 0.5M phosphate buffer, pH 7.4. Sodium radioiodide (I^{125}) aqueous solution (5 µl; 520 µCi/µl) is added to the vial and the vial is stoppered. Freshly diluted chloramine-T solution (20 µl; 0.5 µg/ml) in phosphate buffer (0.5M, pH 7.4) is injected into the vial and the vial is mixed for about 30 seconds and then allowed to stand for an additional 30 seconds. Sodium metabisulfite solution (20 µl; 0.5 µg/µl in 0.5M phosphate buffer, pH 7.4) is injected through the stopper to quench the reaction. The vial is mixed well. The iodination mixture is purified using thin layer chromatography.

Method 2

To an aqueous solution of 5′ - O - [3 - (4 - hydroxyphenyl)glutaroyl] vitamin B_{12} (20 µl; 500 µg/ml) in a glass vial is added 40 µl of 0.5M phosphate buffer, pH 7.4. Sodium radioiodide (I^{125}) aqueous solution (10 µl; 520 µCi/µl) is added to the vial and the vial is stoppered. Freshly diluted chloramine-T solution (20 µl; 2 mg/µl) in phosphate buffer (.5M, pH 7.4) is injected into the vial and the vial is mixed for about 30 seconds and then allowed to stand for an additional 4.5 minutes. Sodium metabisulfite solution (20 µl; 2 µg/µl in 0.5M phosphate buffer, pH 7.4) is injected through the stopper to quench the reaction. The vial is mixed well. The iodination mixture is purified using thin layer chromatography.

Claims

1. A vitamin B_{12} derivative having the formula I



(I)

wherein R is hydrogen or an alkyl group of 1 to 3 carbon atoms and n is 0, 1, 2, 3, or 4.

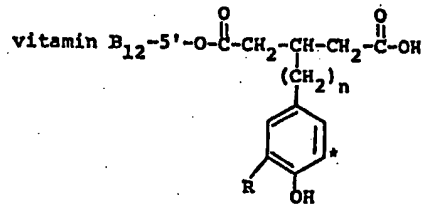
2. A vitamin B_{12} derivative in accordance with Claim 1 wherein n is 0.

3. A vitamin B_{12} derivative in accordance with Claim 1 wherein R is hydrogen.

4. A vitamin B_{12} derivative in accordance with Claim 1 wherein R is an alkyl group of 1 to 3 carbon atoms.

5. 5′-O-[3-(4-Hydroxyphenyl)-glutaroyl]vitamin B_{12}.

6. A radiolabeled vitamin B_{12} derivative having the formula VII

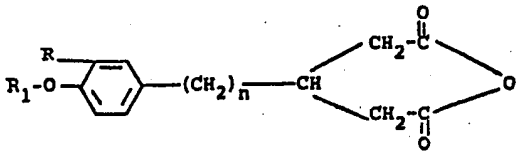


(VII)

wherein R and n have the same meaning as in claim 1 and the asterisk (*) indicates tagging with a radioisotope.

7. A radiolabeled vitamin B_{12} derivative in accordance with Claim 6 wherein the radioisotope is iodine-125.

8. A process for preparing a vitamin B_{12} derivative according to claim 1, characterized by reacting vitamin B_{12} with a compound of the formula II



(II)

wherein R_1 is a protecting group and R and n have the meaning as defined in claim 1, and then removing said protecting group according to conventional methods.

9. A process according to claim 8 wherein R is hydrogen and n is zero.

10. A process for preparing a radiolabeled compound according to claim 6, characterized by labeling the product prepared according to claim 8 with a radioisotope.

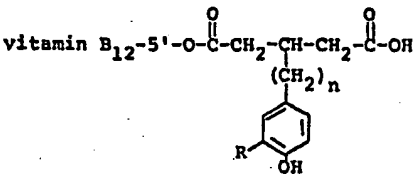
11. A process according to claim 10 wherein the product is radiolabeled with iodine-125.

12. Preparation for inducing antibody formation comprising a vitamin B_{12} derivative according to claims 1 to 5, an immunogenic carrier and optionally an adjuvant.

13. Use of the radiolabeled vitamin B_{12} derivatives according to claims 6 and 7 as tracers in radioimmunoassay procedures.

Patentansprüche

1. Ein Vitamin B_{12}-Derivat der Formel I



(I)

in der R ein Wasserstoffatom oder einen Alkylrest mit 1 bis 3 Kohlenstoffatomen bedeutet und n den Wert 0, 1, 2, 3 oder 4 hat.

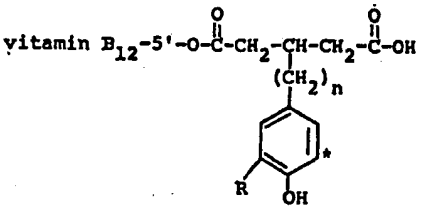
2. Ein Vitamin B_{12}-Derivat nach Anspruch 1, in dem n den Wert 0 hat.

3. Ein Vitamin B_{12}-Derivat nach Anspruch 1, in dem R ein Wasserstoffatom darstellt.

4. Ein tamin B_{12}-Derivat nach Anspruch 1, in dem R einen Alkylrest mit 1 bis 3 Kohlenstoffatomen bedeutet.

5. 5'-O-[3-(4-Hydroxyphenyl)-glutaroyl]-vitamin B_{12}.

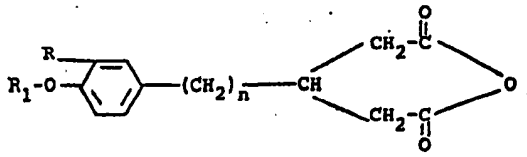
6. Ein radioaktiv markiertes Vitamin B_{12}-Derivat der Formel VII



in der R und n die in Anspruch 1 angegebene Bedeutung haben und das Sternchen (*) eine Markierung mit einem Radioisotop bedeutet.

7. Ein radioaktiv markiertes Vitamin B_{12}-Derivat gemäß Beispiel 6, in dem das radioaktive Isotop Jod-125 ist.

8. Verfahren zur Herstellung eines Vitamin B_{12}-Derivats nach Anspruch 1, dadurch gekennzeichnet, daß man Vitamin B-12 mit einer Verbindung der Formel II



(II)

zur Umsetzung bringt, in der R_1 eine Schutzgruppe und R und n die in Anspruch 1 angegebene Bedeutung haben, worauf man die genannte Schutzgruppe in an sich bekannter Weise entfernt.

9. Verfahren nach Anspruch 8, dadurch gekennzeichnet, daß R ein Wasserstoffatom ist und n den Wert 0 hat.

10. Verfahren zur Herstellung von radioaktiv markierten Verbindungen nach Anspruch 6, dadurch gekennzeichnet, daß man das gemäß Anspruch 8 hergestellte Produkt mit einem radioaktiven Isotop markiert.

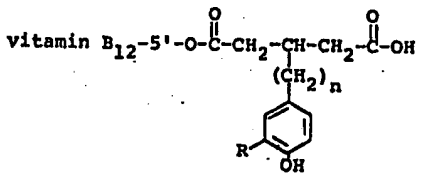
11. Verfahren nach Anspruch 10, dadurch gekennzeichnet, daß man das Produkt mit Jod-125 radioaktiv markiert.

12. Zubereitungen zur Induktion von Antikörperbildung enthaltend ein Vitamin B_{12}-Derivat nach Anspruch 1 bis 5, einen immunogenen Träger und gegebenenfalls einen Hilfsstoff.

13. Verwendung der radioaktiv markierten Vitamin B_{12}-Derivate nach den Ansprüchen 6 und 7 als Tracer bei Radioimmunoassays (Radioimmunotest)-Verfahren.

Revendications

1. Dérivé de la vitamine B_{12}, ayant pour formule:



(I)

dans laquelle R est un atome d'hydrogène ou un radical alkyle de 1 à 3 atomes de carbone, et n est égal à 0, 1, 2, 3 ou 4.

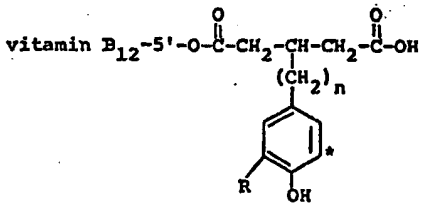
2. Dérivé de la vitamine B_{12} selon la revendication 1, dans la formule duquel n est égal à 0.

3. Dérivé de la vitamine B_{12} selon la revendication 1, dans la formule duquel R est un atome d'hydrogène.

4. Dérivé de la vitamine B_{12} selon la revendication 1, dans la formule duquel R est un radical alkyle de 1 à 3 atomes de carbone.

5. 5'-O-[3-(4-Hydroxyphényl)-glutaroyl]vitamine B_{12}.

6. Dérivé radiomarqué de la vitamine B_{12}, ayant pour formule:

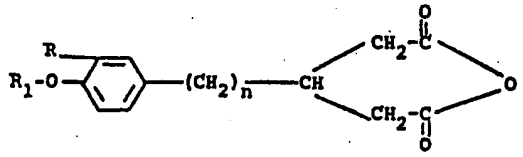


(VII)

dans laquelle R et n ont les mêmes définitions que dans la revendication 1 et l'astérisque (*) indique le marquage avec un radio-isotope.

7. Dérivé radiomarqué de la vitamine B_{12} selon la revendication 6, dans lequel le radio-isotope est l'iode 125.

8. Procédé de préparation d'un dérivé de la vitamine B_{12} selon la revendication 1, caractérisé en ce qu'on fait réagir la vitamine B_{12} avec un composé de formule:



dans laquelle R_1 est un groupement protecteur et R et n ont les mêmes définitions que dans la revendication 1, puis on élimine ledit groupement protecteur par une méthode classique

9. Procédé selon la revendication 8, dans lequel R est un atome d'hydrogène et n est égal à zéro.

10. Procédé de préparation d'un composé radiomarqué selon la revendication 6, caractérisé en ce qu'on marque avec un radio-isotope le produit préparé selon la revendication 8.

11. Procédé selon la revendication 10, caractérisé en ce que le produit est radiomarqué avec de l'iode 125.

12. Préparation permettant d'induire la formation d'anticorps, comprenant un dérivé de la vitamine B_{12} selon les revendications 1 à 5, un porteur immunogénique et facultativement un adjuvant.

13. Utilisation des dérivés radiomarqués de la vitamine B_{12} selon les revendications 6 et 7, comme traceurs on marqueurs dans les modes opératoires de dosage radioimmunologique.

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